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<p>(21) International Application Number: PCT/US95/03930</p> <p>(22) International Filing Date: 5 April 1995 (05.04.95)</p> <p>(30) Priority Data: 08/222,956 5 April 1994 (05.04.94) US</p> <p>(71) Applicants (for all designated States except US): EXPONENTIAL BIOTHERAPIES, INC. [US/US]; 19 West 34th Street, Penthouse, New York, NY 10001 (US). THE UNITED STATES OF AMERICA, represented by THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Washington, DC 20032 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): CARLTON, Richard, M. [US/US]; 3 Secor Drive, Port Washington, NY 11050 (US). MERRIL, Carl, R. [US/US]; 2 Winder Court, Rockville, MD 20850 (US). ADHYA, Sankar, L. [US/US]; 14400 Kings Grant Street, Gaithersburg, MD 20878 (US).</p> <p>(74) Agents: MURRAY, Robert, B. et al.; Nikaido, Marmelstein, Murray &amp; Oram, Metropolitan Square, Suite 330 - G Street Lobby, 655 15th Street, N.W., Washington, DC 20005-5701 (US).</p>		<p>(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN; European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).</p> <p>Published With international search report.</p>
(54) Title: ANTIBACTERIAL THERAPY WITH GENOTYPICALLY MODIFIED BACTERIOPHAGE		
<p>(57) Abstract</p> <p>The present invention is directed to bacteriophage therapy, using methods that enable the bacteriophage to delay inactivation by any and all parts of the host defense system (HDS) against foreign objects that would tend to reduce the numbers of bacteriophage and/or the efficiency of those phage at killing the host bacteria in an infection. Disclosed is a method of producing bacteriophage modified for anti-HDS purposes, one method being selection by serial passaging, and the other method being genetic engineering of a bacteriophage, so that the modified bacteriophage will remain active in the body for longer periods of time than the wild-type phage.</p>		

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"Antibacterial Therapy with Genotypically Modified Bacteriophage"

FIELD OF THE INVENTION

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The present invention relates to a method of delaying the inactivation of bacteriophages by an animal's host defense system. One method of delaying inactivation is the use of novel bacteriophages whose genomes have been modified. Methods useful for  
10 modifying the bacteriophage genome include but are not limited to selection of mutant strains by serial passage, and the creation of new strains by genetic engineering. Such novel bacteriophages have the ability to delay being sequestered by, engulfed by, or otherwise inactivated by one or more of the processes of an  
15 animal's host defense system (HDS). This novel attribute allows the "anti-HDS modified" bacteriophage to have a longer survival time in an animal's body than the corresponding wild-type bacteriophage, and that in turn allows the modified phage to be more effective than the wild-type phage at treating (or assisting  
20 in the treatment of) a bacterial infection.

The present invention also is directed to specific methods of using bacteriophages for treating infectious bacterial diseases. The route of administration can be by any means including delivering the phage by aerosol to the lungs.

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### BACKGROUND OF THE INVENTION

In the 1920s, shortly after the discovery of bacterial viruses (bacteriophages), the medical community began to extensively pursue the treatment of bacterial diseases with bacteriophage therapy. The idea of using phage as a therapy for infectious bacterial diseases was first proposed by d'Herelle in 1918, as a logical application of the bacteriophages' known ability to invade and destroy bacteria. Although early reports of bacteriophage therapy were somewhat favorable, with continued clinical usage it became clear that this form of therapy was inconsistent and unpredictable in its results. Disappointment with phage as a means of therapy grew, because the great potential of these viruses to kill bacteria in vitro was not realized in vivo. This led to a decline in attempts to develop clinical usage of phage therapy, and that decline accelerated once antibiotics began to be introduced in the 1940s and 50s. From the 1960s to the present, some researchers who adopted certain bacteriophages as a laboratory tool and founded the field of molecular biology have speculated as to why phage therapy failed.

Despite the general failure of phage as therapy, isolated groups of physicians have continued to try to use these agents to treat infectious diseases. Many of these efforts have been concentrated in Russia and India, where the high costs of and lack of availability of antibiotics continues to stimulate a search for alternative therapies. See for example Vogovazova et al.

"Effectiveness of Klebsiella pneumoniae Bacteriophage in the Treatment of Experimental Klebsiella Infection", Zhurnal Mikrobiologii, Epidemiologii Immunobiologii, pp. 5-8 (April, 1991); and Vogovazova et al., "Immunological Properties and Therapeutic Effectiveness of Preparations of Klebsiella Bacteriophages", Zhurnal Mikrobiologii, Epidemiologii Immunobiologii, pp. 30-33 (March, 1992)]. These articles are similar to most of the studies of phage therapy, including the first reports by d'Herelle, in that they lack many of the controls required by researchers who investigate anti-infectious therapies. In addition, these studies often have little or no quantification of clinical results. For example, in the second of the two Russian articles cited above, the Results section concerning Klebsiella phage therapy states that "Its use was effective in...ozena (38 patients), suppuration of the nasal sinus (5 patients) and of the middle ear (4 patients)...In all cases a positive clinical effect was achieved without side effects from the administration of the preparation". Unfortunately, there were no placebo controls or antibiotic controls, and no criteria were given for "improvement".

Another clinical use of phage that was developed in the 1950s and is currently still employed albeit to a limited extent, is the use of phage lysate, specifically staphphage lysate (SPL). The researchers in this field claim that a nonspecific, cell-mediated immune response to staph endotoxin is an integral and essential part of the claimed efficacy of the SPL. [See, eg., Esber et al., J. Immunopharmacol., Vol. 3, No. 1, pp. 79-92 (1981); Aoki et al.,

Augmenting Agents in Cancer Therapy (Raven, New York), pp. 101-112 (1981); and Mudd et al., Ann. NY Acad. Sci., Vol. 236, pp. 244-251 (1974).] In this treatment, it seems that the purpose of using the phage is to lyse the bacteria specifically to obtain bacterial antigens, in a manner that those authors find preferential to lysing by sonication or other physical/chemical means. Here again, some difficulties arise in assessing these reports in the literature, because, in general, there are no placebo controls and no standard antibiotic controls against which to measure the reported efficacy of the SPL. More significantly, there is no suggestion in these articles to use phage per se in the treatment of bacterial diseases. Moreover, the articles do not suggest that phage should be modified in any manner that would delay the capture/sequestration of phage by the host defense system.

Since many patients will recover spontaneously from infections, studies must have carefully designed controls and explicit criteria to confirm that a new agent is effective. The lack of quantification and of controls in most of the phage reports from d'Herelle on makes it difficult if not impossible to determine if the phage therapies have had any beneficial effect.

As there are numerous reports of attempts at phage therapy, one would assume that had it been effective, it would have flourished in the period before antibiotics were introduced. But phage therapy has been virtually abandoned, except for the isolated pockets mentioned above.

As noted above, some of the founders of molecular biology who

pioneered the use of specific phages to investigate the molecular basis of life processes have speculated as to why phage therapy was not effective. For example, G. Stent in his book Molecular Biology of Bacterial Viruses, WH Freeman & Co. (1963) pp. 8-9, stated the following:

"Just why bacteriophages, so virulent in their antibiotic action in vitro, proved to be so impotent in vivo, has never been adequately explained. Possibly the immediate antibody response of the patient against the phage protein upon hypodermic injection, the sensitivity of the phage to inactivation by gastric juices upon oral administration, and the facility with which bacteria acquire immunity or sport resistance against phage, all militated against the success of phage therapy."

In 1973, one of the present inventors, Dr. Carl Merrill, discovered along with his coworkers that phage lambda, administered by various routes (per os, IV, IM, IP) to germ-free, non-immune mice, was cleared out of the blood stream very rapidly by the organs of the reticulo-endothelial system, such as the spleen, liver and bone marrow. [See Geier, Trigg and Merrill, "Fate of Bacteriophage Lambda in Non-Immune, Germ-Free Mice", Nature, 246, pp. 221-222 (1973).] These observations led Dr. Merrill and his coworkers to suggest (in that same Nature article cited above) overcoming the problem by flooding the body with colloidal particles, so that the reticulo-endothelial system would be so overwhelmed engulfing the particles that the phage might escape capture. Dr.

Merril and his coworkers did not pursue that approach at the time as there was very little demand for an alternative antibacterial treatment such as phage therapy in the 1970s, given the numerous and efficacious antibiotics available.

5 Subsequently, however, numerous bacterial pathogens of great importance to mankind have become multi-drug resistant (MDR), and these MDR strains have spread rapidly around the world. As a result, hundreds of thousands of people now die each year from infections that could have been successfully treated by antibiotics  
10 just 4-5 years ago. [See e.g. C. Kunin, "Resistance to Antimicrobial Drugs - A Worldwide Calamity", *Annals of Internal Medicine*, 1993;118:557-561; and H. Neu, "The Crisis in Antibiotic Resistance", *Science* 257, 21 Aug 1992, pp. 1064-73.] In the case of MDR tuberculosis, e.g., immunocompromised as well as non-  
15 immunocompromised patients in our era are dying within the first month or so after the onset of symptoms, despite the use of as many as 11 different antibiotics.

Medical authorities have described multi-drug resistance not just for TB, but for a wide variety of other infections as well.  
20 Some infectious disease experts have termed this situation a "global crisis". A search is underway for alternative modes and novel mechanisms for treating these MDR bacterial infections.

Bacteriophage therapy offers one possible alternative treatment. Learning from the failure of bacteriophage therapy in  
25 the past, the present inventors have discovered effective ways to overcome the major obstacles that were the cause of that failure.



One object of the present invention is to develop novel bacteriophages which are able to delay inactivation by an animal's host defense system, any component of which may be diminishing the numbers or the efficacy of the phage that have been administered.

Another object of the present invention is to develop a method for treating bacterial infectious diseases in an animal by administering to the animal an effective amount of the novel bacteriophage, and by an appropriate route of administration.

#### SUMMARY OF THE INVENTION

In the present invention, novel bacteriophages are developed by serial passage or by genetic engineering, to obtain bacteriophages capable of delaying inactivation by any component of an animal's host defense system (HDS) against foreign bodies. This allows the novel phages to survive for longer periods of time in the circulation and the tissues than the wild-type phage, thereby prolonging viability and making these modified phages more efficient at reaching and invading the bacteria at the site of an infection.

The administration of an anti-HDS phage that has been developed by serial passage or by genetic engineering will enable the animal recipient to efficaciously fight an infection with the corresponding bacterial pathogen. The phage therapy of this invention will therefore be useful either as an adjunct to standard anti-infective therapies, or as a stand-alone therapy.

The phages of the present invention can be administered by any route, such as oral, pulmonary (by aerosol or by other respiratory device for respiratory tract infections), nasal, IV, IP, per vagina, per rectum, intra-ocular, by lumbar puncture, intrathecal, and by burr hole or craniotomy if need be for direct insertion onto the meninges (e.g. in a heavily thickened and rapidly fatal tuberculous meningitis).

#### DETAILED DESCRIPTION OF THE INVENTION

One of the major obstacles to bacteriophage therapy is the fact that when phages are administered to animals, they are rapidly eliminated by the animal's HDS. That suggests that the phages are not viable in the animal's circulation or tissues for a long enough time to reach the site of infection and invade the bacteria. Thus, the object of the present invention is to develop bacteriophages that are able to delay inactivation by the HDS. This will prolong phage viability in the body.

The term "host defense system" as used herein refers to all of the various structures and functions that help an animal to eliminate foreign bodies. These defenses include but are not limited to the formed cells of the immune system and the humoral components of the immune system, those humoral components including such substances as complement, lysozymes and beta-lysin. In addition, the organs of what has often been referred to as the "reticulo-endothelial system" (spleen, liver, bone marrow, lymph

glands, etc.) also serve as part of the host defense system. In addition to all the phenomena cited just above which take place within this "reticulo-endothelial system", there has also been described a sequestering action wherein foreign materials (specifically including bacteriophage) are captured non-phagocytically and non-destructively in the spleen by what is known as the Schweigger-Seidel capillary sheaths - a phenomenon that may or may not involve antigen capture [See e.g. Nagy, Z., Horrath, E., and Urban, Z., Nature New Biology, 242: p. 241 (1973).]

10 The phrase "substantially eliminate" as used regarding the present invention, indicates that the number of bacteria is reduced to a number which can be completely eliminated by the animal's defense system or by using conventional antibacterial therapies.

15 Enabling bacteriophages to delay inactivation by those host defense systems - whichever components of it may or may not be employed in any given case - would be likely to result in an increased in vivo killing of bacterial pathogens that are in the host range for those bacteriophages.

20 In one embodiment, bacteriophages are selected by serial passage. These will by their nature have a delay in their inactivation by the HDS. Essentially, the serial passage is accomplished by administering the phage to an animal and obtaining serial blood samples over an extended period of time. Eventually one obtains viable phage that are able to delay inactivation by the HDS. When a period is reached where in blood samples there remains 25 0.01%-1.0%, and preferably 0.1%, of the number of phages originally

placed in circulation, a sample of this remaining phage is grown up to sufficiently high titer to be injected into a second animal of the same species. [For methods of clonal purification, see M. Adams, Bacteriophages, Interscience Publishers, pp. 454-460 (1959)]. Serial blood samples are again obtained over time, and the process described above is repeated iteratively so that each time when approximately 0.1% of the phages are left, it takes longer and longer with each serial passage to reach that point when only 0.1% of the phage administered still remain in circulation. By this method of clonal purification and selection, a phage strain will be isolated that can survive at least 15 % longer in the body than the longest-surviving wild-type phage.

After a number of serial passages of these non-mutagenized or mutagenized (see below) bacteriophage, a prototype "anti-HDS modified" bacteriophage is obtained. As used herein, an "anti-HDS modified" phage is defined as any phage (whether modified by serial passage or by genetic engineering) that has a half-life within the animal that is at least 15% greater than the half-life of the original wild-type phage from which it was derived. Half-life refers to the point in time when out of an initial IV dose (e.g.  $1 \times 10^{12}$ ) of a given phage, half ( $1 \times 10^6$ ) of them still remain in circulation, as determined by serial pfu experiments ("pfu" meaning plaque forming units, a convenient measure of how many phage are present in a given sample being assayed). A 15% longer half-life indicates a successful delay of inactivation by the HDS.

The evidence that the HDS-evading phages do in fact remain

5 viable for a longer period of time in the body is obtained by demonstrating not only by the longer time that they remain in the circulation, but also by the higher numbers of them that remain in the circulation at a given point in time. This slower rate of clearance is demonstrated by the fact that ten minutes after the IV injection of  $1 \times 10^{12}$  of the phages into a test animal, the number of the phages still in circulation (as measured by pfu assays) is at least 10% higher than the number of the corresponding wild-type phage still in circulation in the control animal, at that point in time.

10 Instead of awaiting the spontaneous mutations that are selected for in the above method, alternatively mutations can be provoked during the growth of the phage in its host bacteria. The mutations may produce specimens of phage that, after selection by serial passage, are even more efficient than the non-mutagenized phage at delaying inactivation by the host defense system. Mutagenization is achieved by subjecting the phage to various stimuli, such as, but not limited to, acridine compounds, ethidium bromide in the presence of light, radioactive phosphorus, and various forms of radiation (X-rays, UV light, etc.). Mutants resulting from the iterative procedure described above, and that are found to have a longer survival time than the wild-type phage, are grown to high titer and are used to treat infectious diseases in animals and in humans.

25 The phage obtained by the above methods are referred to as "anti-HDS selected".

An altogether different method to achieve the desired result is to genetically engineer a phage so that it expresses molecules on its surface coat, where said molecules antagonize, inactivate, or in some other manner impede those actions of the HDS that would otherwise reduce the viability of the administered phages. One of the ways to accomplish this is to engineer a phage to express molecules that antagonize one or more of the complement components.

Complement components fix to bacteriophages, and these bacteriophages then adhere to certain white blood cells (such as macrophages) that express complement receptors. Numerous peptides have been synthesized that antagonize the functions of the various complement components. [See e.g. Lambris, J.D. et al, "Use of synthetic peptides in exploring and modifying complement reactivities" in Activators and Inhibitors of Complement, ed. R. Sim, Kluwer Academic Publishers, Boston, 1993.] Lambris et al. (op.cit.) cite "a series of synthetic peptides spanning the convertase cleavage site in C3 (that are) found to inhibit complement activation by both the classical and alternative pathways". Among the peptides cited is a six amino acid peptide (LARSNL, residues 746-751 of C3) that "inhibits both pathways equally well".

In one method of genetically engineering such a phage, a fusion protein is obtained, wherein the peptide will be bound to the carboxyl end of the surface protein of interest [See e.g. Sambrook, J., Fritsch, E., and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press,

Cold Spring Harbor, NY, 1989]. This construct is made by cloning the gene for the phage surface protein into a plasmid vector system, and then cloning the oligonucleotide for the peptide of interest into this carrying vector by in-frame fusion at the 3'-end of the gene for the surface protein. This fusion of the gene for the phage surface protein with the oligonucleotide for the complement-antagonizing peptide would then be incorporated into the phage of interest by the in vivo generalized recombination system in the host bacteria for the phage of interest. Phage whose genomic sequence is already completely known, and phage whose genomic sequence is unknown or partially unknown can be used in the present invention.

The surface expression of a recombinant complement-antagonizing peptide is but one example of several complement-related strategies that might be used for these purposes. Another example would be the expression of a human complement-antagonizing protein on the surface of a phage. Several transplantation research facilities are currently expressing such human complement-antagonizing proteins in transgenic animals, in the hopes that when these transgenic organs are donated they will not be immunologically rejected by a human recipient. [See e.g. Genetic Engineering News, October 15, 1993, p.1.] In an analogous manner, the expression of such recombinant human complement-antagonizing proteins on the surface of a bacteriophage may allow the phage to delay being inactivated by the host defense system.

In addition to complement-related strategies, there are many

other categories of molecules that can be recombinantly engineered into a phage to delay inactivation by the host's defense system. Other categories of molecules that could be expressed on the surface of bacteriophages, and would fall under the scope of this invention, include but are not limited to: interleukins and other cytokines; autocrines; and inhibitors of the various cellular activating or inhibiting factors (e.g. inhibitors of macrophage activating factor). Genes for these proteins (or for active subunits of them) can be incorporated into a phage genome so that they will be expressed on the surface.

In addition, if it were possible to get a given bacterial host strain to glycosylate a recombinant protein, then the purpose of the invention could be served by introducing genes that will express glycosylated proteins. Such proteins are known by their negative charge to repel immune cells, such as the macrophage. Examples might include but would not be limited to (1) the C-terminal portion of the  $\beta$ -subunit of human chorionic gonadotrophin (hCG), and (2) the various glycoporphins on the surfaces of blood cells.

Phage modified in this manner are referred to as "anti-HDS engineered".

The present invention can be applied across the spectrum of bacterial diseases, either by serial passage of phages (mutagenized or non-mutagenized) or by genetically engineering phages, so that phages are developed that are specific for each of the bacterial strains of interest. In that way, a full array of anti-HDS



selected and/or anti-HDS engineered bacteriophage is developed for virtually all the bacterial (and other applicable) pathogens for man, his pets, livestock and zoo animals (whether mammal, avian, or pisciculture). Phage therapy will then be available:

5        1) as an adjunct to or as a replacement for those antibiotics and/or chemotherapeutic drugs that are no longer functioning in a bacteriostatic or bactericidal manner due to the development of multi-drug resistance;

10       2) as a treatment for those patients who are allergic to the antibiotics and/or chemotherapeutic drugs that would otherwise be indicated; and

      3) as a treatment that has fewer side effects than many of the antibiotics and/or chemotherapeutic drugs that would otherwise be indicated for a given infection.

15       The second embodiment of the present invention is the development of methods to treat bacterial infections in animals through phage therapy with the anti-HDS modified bacteriophages described above. Hundreds of bacteriophages and the bacterial species they infect are known in the art. The present invention is  
20       not limited to a specific bacteriophage or a specific bacteria. Rather, the present invention can be utilized to develop anti-HDS modified bacteriophages which can be used to treat any and all infections caused by their host bacteria.

25       While it is contemplated that the present invention can be used to treat any bacterial infection in an animal, it is particularly contemplated that the methods described herein will be

very useful as a therapy (adjunctive or stand-alone) in infections caused by drug-resistant bacteria. Experts report [See e.g. Gibbons, A., "Exploring New Strategies to Fight Drug-Resistant Microbes, Science, 21 Aug. 1993, pp. 1036-38.] that at the present time, the drug-resistant bacterial species and strains listed below represent the greatest threat to mankind:

1. All of the clinically important members of the family Enterobacteriaceae, most notably but not limited to the following:

- a) All the clinically important strains of *Escherichia*, most notably *E. coli*. One among a number of candidate wild-type phages against these particular pathogens that could be used as a starting point for the serial passage and/or the genetic engineering of the present invention would be ATCC phage # 23723-B2. [Note: For purposes of brevity, in all the following examples of pathogens, the corresponding wild-type phage will be indicated by the following phraseology: "Example of corresponding phage: \_\_\_\_\_".]
- b) All the clinically important strains of *Klebsiella*, most notably *K. pneumoniae* [Example of corresponding phage: ATCC phage # 23356-B1].
- c) All the clinically important strains of *Shigella*, most notably *S. dysenteriae* [Example of corresponding phage: ATCC phage # 11456a-B1].
- d) All the clinically important strains of *Salmonella*, including *S. abortus-equus* [Example of corresponding phage: ATCC phage # 9842-B1], *S. typhi* [Example of corresponding phage: ATCC phage

- 5       # 19937-B1], S. typhimurium [Example of corresponding phage: ATCC phage # 19585-B1], S. newport [Example of corresponding phage: ATCC phage # 27869-B1], S. paratyphi-A [Example of corresponding phage: ATCC phage # 12176-B1], S. paratyphi-B [Example of corresponding phage: ATCC phage # 19940-B1], S. potsdam [Example of corresponding phage: ATCC phage # 25957-B2], and S. pollorum [Example of corresponding phage: ATCC phage # 19945-B1].
- 10       e) All the clinically important strains of Serratia, most notably S. marcescens [Example of corresponding phage: ATCC phage # 14764-B1].
- f) All the clinically important strains of Yersinia, most notably Y. pestis [Example of corresponding phage: ATCC phage # 11953-B1].
- 15       g) All the clinically important strains of Enterobacter, most notably E. cloacae [Example of corresponding phage: ATCC phage # 23355-B1].
- 20       2. All the clinically important Enterococci, most notably E. faecalis [Example of corresponding phage: ATCC phage # 19948-B1] and E. faecium [Example of corresponding phage: ATCC phage #19953-B1].
- 25       3. All the clinically important Haemophilus strains, most notably H. influenzae [a corresponding phage is not available from ATCC for this pathogen, but several can be obtained from WHO or other labs that make them available publicly].

4. All the clinically important Mycobacteria, most notably M. tuberculosis [Example of corresponding phage: ATCC phage # 25618-B1], M. avium-intracellulare, M. bovis, and M. leprae. [Corresponding phage for these pathogens are available commercially from WHO, via The National Institute of Public  
5 Healthy & Environmental Protection, Bilthoven, The Netherlands].
5. Neisseria gonorrhoeae and N. meningitidis [Corresponding phage for both can be obtained publicly from WHO or other sources].
- 10 6. All the clinically important Pseudomonads, most notably P. aeruginosa [Example of corresponding phage: ATCC phage # 14203-B1].
7. All the clinically important Staphylococci, most notably S. aureus [Example of corresponding phage: ATCC phage # 27690-B1] and S. epidermidis [Corresponding phage are available  
15 publicly through the WHO, via the Colindale Institute in London].
8. All the clinically important Streptococci, most notably S. pneumoniae [Corresponding phage can be obtained publicly  
20 from WHO or other sources].
9. Vibrio cholera [Example of corresponding phage: ATCC phage # 14100-B1].

25 There are additional bacterial pathogens too numerous to mention that, while not currently in the state of antibiotic-resistance crisis, nevertheless make excellent candidates for

treatment with anti-HDS modified bacteriophages that are able to delay inactivation by the HDS, in accordance with the present invention. Thus, all bacterial infections caused by bacteria for which there is a corresponding phage can be treated using the present invention.

Any phage strain capable of doing direct or indirect harm to a bacteria (or other pathogen) is contemplated as useful in the present invention. Thus, phages that are lytic, phages that are lysogenic but can later become lytic, and nonlytic phages that can deliver a product that will be harmful to the bacteria are all useful in the present invention.

The animals to be treated by the methods of the present invention include but are not limited to man, his domestic pets, livestock, pisciculture, and the animals in zoos and aquatic parks (such as whales and dolphins).

The anti-HDS modified bacteriophage of the present invention can be used as a stand-alone therapy or as an adjunctive therapy for the treatment of bacterial infections. Numerous antimicrobial agents (including antibiotics and chemotherapeutic agents) are known in the art which would be useful in combination with anti-HDS modified bacteriophage for treating bacterial infections. Examples of suitable antimicrobial agents and the bacterial infections which can be treated with the specified antimicrobial agents are listed below. However, the present invention is not limited to the antimicrobial agents listed below as one skilled in the art could easily determine other antimicrobial agents useful in combination

with anti-HDS modified bacteriophage.

<u>Pathogen</u>	<u>Antimicrobial or antimicrobial group</u>
E. <u>coli</u>	
5     -uncomplicated urinary tract infection	trimethoprim-sulfamethoxazole (abbrev. TMO-SMO), or ampicillin; 1st generation cephalosporins, ciprofloxacin
10    -systemic infection	ampicillin, or a 3rd generation cephalosporin; aminoglycosides, aztreonam, or a penicillin + a penicillinase inhibitor
15    Klebsiella <u>pneumoniae</u>	1st generation cephalosporins; 3rd gener. cephalosporins, cefotaxime, moxalactam, amikacin, chloramphenicol
20    Shigella (various)	ciprofloxacin; TMO-SMO, ampicillin, chloramphenicol
Salmonella:	
-S. <u>typhi</u>	chloramphenicol; ampicillin or TMO-SMO
25    -non- <u>typhi</u> species	ampicillin; chloramphenicol, TMO-SMO, ciprofloxacin

- Yersinia pestis streptomycin; tetracycline,  
chloramphenicol
- 5 Enterobacter cloacae 3rd generation cephalosporins,  
gentamicin, or tobramycin;  
carbenicillin, amikacin, aztreonam,  
imipenem
- 10 Hemophilus influenzae:  
-meningitis chloramphenicol or 3rd generation  
cephalosporins; ampicillin
- other H. infl. infections ampicillin; TMO-SMO, cefaclor,  
cefuroxime, ciprofloxacin
- 15 Mycobacterium tuberculosis isoniazid (INH) + rifampin or  
and M. avium-intracellulare rifabutin, the above given along with  
pyrazinamide +/- ethambutol
- 20 Neisseria:  
-N. meningitidis penicillin G; chloramphenicol, or a  
sulfonamide
- N. gonorrhoeae:  
25 penicillin-sensitive penicillin G; spectinomycin,  
ceftriaxone

penicillin-resistant

ceftriaxone; spectinomycin,  
cefuroxime or cefoxitin,  
ciprofloxacin

5 *Pseudomonas aeruginosa*

tobramycin or gentamycin (+/- carben-  
icillin, aminoglycosides); amikacin,  
ceftazidime, aztreonam, imipenem

*Staph aureus*

10 -non-penicillinase  
producing

penicillin G; 1st generation  
cephalosporins, vancomycin, imipenem,  
erythromycin

-penicillinase producing

15

*Streptococcus pneumoniae*

a penicillinase-resisting penicillin;  
1st generation cephalosporins, vanco-  
mycin, imipenem, erythromycin  
penicillin G; 1st generation  
cephalosporins, erythromycin,  
chloramphenicol

20 *Vibrio cholera*

tetracycline; TMO-SMO

25 The routes of administration include but are not limited to:  
oral, aerosol or other device for delivery to the lungs, nasal  
spray, intravenous, intramuscular, intraperitoneal, intrathecal,



vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. The dosage of administration is contemplated to be in the range of about  $10^6$  to about  $10^{13}$  pfu/per kg/per day, and preferably about  $10^{12}$  pfu/per kg/per day. The phage are administered until successful elimination of the pathogenic bacteria is achieved.

With respect to the aerosol administration to the lungs, the anti-HDS modified phage is incorporated into an aerosol formulation specifically designed for administration to the lungs by inhalation. Many such aerosols are known in the art, and the present invention is not limited to any particular formulation. An example of such an aerosol is the Proventil<sup>™</sup> inhaler manufactured by Schering-Plough, the propellant of which contains trichloromonofluoromethane, dichlorodifluoromethane and oleic acid. The concentrations of the propellant ingredients and emulsifiers are adjusted if necessary based on the phage being used in the treatment. The number of phage to be administered per aerosol treatment will be in the range of  $10^6$  to  $10^{13}$  pfu, and preferably  $10^{12}$  pfu.

The foregoing embodiments of the present invention are further described in the following Examples. However, the present invention is not limited by the Examples, and variations will be

apparent to those skilled in the art without departing from the scope of the present invention. In particular, any bacteria and phage known to infect said bacteria can be substituted in the experiments of the following examples.

5

### EXAMPLES

Example 1: Selection of anti-HDS selected phage by serial passage through mice.

10 Part 1. A stock of mutagenized or non-mutagenized lambda coliphage strain is injected in one bolus into the blood of laboratory mice at  $10^{12}$  pfu/per kg, suspended in 0.5 cc of sterile normal saline. The mice are periodically bled to follow the survival of the phage in the body. The phage are assayed by  
15 plating them on their laboratory host, E. coli. When the titer of phage in the mice reaches a range of 0.01%-1.0%, and preferably 0.1%, of the titer initially injected, the phage isolated at this point in time are plaque isolated and the procedure repeated. The repeated passage of the lambda phage between animal and bacteria  
20 yields a phage strain that has a longer survival time in the body of the mice. The anti-HDS selected phage strain is then subjected to clonal (plaque) purification.

Where the phage being administered for serial passage have first been mutagenized, the mutagenization is carried out according  
25 to procedures known in the art [See e.g., Adams, M. Bacteriophages. NY: Wiley Interscience, 1959, pp. 310-318 and pp. 518-520.] For

mutagenization by ultraviolet radiation, during the last 40%-90% (and preferably 65%) of the latent period, the phage (inside the infected host bacteria) are exposed to 3,000-6,000 ergs (and preferably 4,500 ergs) of ultraviolet radiation per square mm. For  
5 mutagenization by X-radiation, a wavelength of 0.95 Å is used at doses from 10-250 (and preferably 150) kiloroentgens.

Example 2: Determination that HDS inactivation is delayed for the  
10 Anti-HDS Selected Phage as compared to Wild-Type Phage.

Two groups of mice are injected with phage as specified below:

Group 1: The experimental group receives an IV injection consisting of  $1 \times 10^{12}$  of the anti-HDS selected phage, suspended in  
15 0.5cc of normal sterile saline.

Group 2: The control group receives a IV injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the serially-passaged phage were derived, suspended in 0.5cc of sterile normal saline.

Both groups of mice are bled at regular intervals, and the  
20 blood samples assayed for phage content (by pfu assays) to determine the following:

1) Assays for half-lives of the two phages: For each group of mice, the point in time is noted at which there remains in circulation only half (i.e.,  $1 \times 10^6$ ) the amount of phage as  
25 administered at the outset. The point in time at which half of the anti-HDS selected phage have been eliminated from the circulation

is at least 15% longer than the corresponding point in time at which half of the wild-type phage have been eliminated from the circulation.

2) Assays for absolute numbers: For each group of mice, a sample of blood is taken at precisely 1 hour after administration of the phage. At 1 hour post-injection, the numbers of anti-HDS-selected phage in circulation are at least 10% higher than the numbers of wild-type phage still in circulation.

10 Example 3: Determination that the anti-HDS selected phage has a greater capacity than wild-type phage to prevent lethal infections in mice.

15 Part 1. Peritonitis Model: An LD<sub>50</sub> dosage of E. coli is administered intraperitoneally (IP) to laboratory mice. The strain of E. coli used is known to be lysed by the coliphage strain that is selected by Serial Passage. The treatment modality is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist  
20 of the following:

Group 1: The experimental group receives an IP injection consisting of  $1 \times 10^{12}$  of the anti-HDS selected phage lambda coliphage suspended in 2cc of sterile normal saline.

25 Group 2: A first control group receives an IP injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the anti-HDS selected phage were developed, suspended in 2cc of normal sterile

saline.

Group 3: A second control group receives an IP injection of 2cc of normal sterile saline.

Evidence that treatment with the anti-HDS selected phage prevented the development of a lethal event in the peritonitis model is measured by using the following three criteria:

(1) Survival of the animal

(2) Bacterial counts: Samples of peritoneal fluid are withdrawn every 1/2 hour from the three groups of infected mice, and the rate of increase or decrease in E. coli colony counts in the three groups is noted

(3) Phage control: Using the samples of IP fluid withdrawn from the infected mice, the numbers of pfu of the anti-HDS selected phage and the numbers of pfu of the wild-type phage are noted.

## Part 2. Bacteremia Model:

An LD<sub>50</sub> dosage of E. coli is administered intravenously (IV) to laboratory mice, where the strain of E. coli used is known to be lysed by the coliphage strain that was chosen for the serial passage. The treatment modality (see below) is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist of the following:

Group 1: The experimental group receives an IV injection consisting of  $1 \times 10^{12}$  of the anti-HDS selected lambda coliphage suspended in 0.5cc of sterile normal saline.

Group 2: A first control group receives an IV injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the Anti-HDS selected phage were developed, suspended in 0.5cc of normal sterile saline.

5      Group 3: A second control group receives an IV injection of 0.5cc of normal sterile saline.

Evidence that treatment with the anti-HDS selected phage prevented the development of a lethal event in the bacteremia model is measured using the following three criteria:

10      (1) Survival of the animal

(2) Bacterial counts: Samples of blood are withdrawn every 1/2 hour from the three groups of infected mice, and the rate of increase or decrease in E. coli colony counts in the three groups is noted.

15      (3) Phage counts: In the samples of blood withdrawn from the infected mice, the numbers of pfu of the anti-HDS selected phage and the numbers of pfu of the wild-type phage are noted.

20      Example 4: Genetic engineering of phage to express molecules that antagonize the host defense system, thereby enabling the phage to delay inactivation by the host defense system.

#### Part 1. Making the Fusion Protein

25      Step 1. A double-stranded DNA encoding the complement antagonizing peptide LARSNL is synthesized on an automated oligonucleotide

synthesizer using standard techniques.

Step 2. The gene for the phage coat surface protein of interest (see part 2, below) is cloned into a plasmid vector system, by techniques known in the art. The oligonucleotide that has been prepared in Step 1 is cloned into the plasmid vector system by in-frame fusion at the 3'-end of the gene for the surface protein.

Step 3. The fusion gene is then incorporated into a phage by the in vivo generalized recombination system in the host bacteria for the phage. The phage then expresses the fusion protein on its surface.

Part 2: Selecting phage coat surface proteins for fusion with the peptide/protein of interest.

A. Incorporating the gene for the complement-antagonizing peptide into a phage whose genome is well characterized

The orfX gene, which encodes a carboxy-terminal tail protein of lambda coliphage, is one for which it is known that foreign nucleotide sequences can be introduced without there being disruption of the structure or function of the phage. The tail surface protein expressed by the orfX gene is made into a fusion protein with the complement-antagonizing peptide, by the plasmid vector method described in part 1 above.

B. Incorporating a gene for a complement-antagonizing peptide into a phage whose genome is not well characterized.

Step 1. Selection of the phage surface protein to be fused with the complement-antagonizing peptide:

a) Isolation of phage coat surface proteins and preparation of antibodies thereto:

(1) Samples of the phage of interest are broken up in 0.1% SDS detergent for 2 minutes at 95°C. The mixture is cooled and placed in 9M urea, and is then separated by high resolution 2D gel electrophoresis. The protein fragments are then isolated from the gel, and processed as described below.

(2) Samples of the protein fragments from the gel are injected into animals to produce either polyclonal or monoclonal antibodies.

(3) Antibodies are isolated and then marked with uranium. These marked antibodies are reacted against whole phage. The marker pinpoints precisely those proteins on the surface of the phage to which the antibodies have bound through visualization by electronmicroscopy. [See e.g. K. Williams and M. Chase, ed., Methods In Immunology and Immunochemistry, Vol.1, 1967, Academic Press.] Antibodies directed against a surface protein extending outward from the surface of the virus are retained for further use.



b) Preparation of phage restriction fragments:

The genome of the phage is cut by restriction enzymes, and the resulting restriction fragments are cloned into expression vector plasmids. Each of these plasmids expresses its corresponding protein, creating a pool of expressed proteins.

c) Reacting the expressed proteins with the marked antibodies:

The antibodies directed against a surface protein extending outward from the surface of the virus are reacted against the proteins expressed by the plasmid vectors.

d) Correlating coat protein antibodies to the plasmid vectors that express the genes for those coat proteins:

The reaction of a marked antibody with an expressed protein pinpoints the expression plasmid whose enclosed restriction fragment expresses the particular protein. Thus, the genomic fragment encoding each coat surface protein is determined using the marked antibodies.

e) Determining that the gene in its entirety has been obtained:

The restriction fragments containing a gene for a surface protein are micro-sequenced by the Sanger technique to determine

- (1) the precise amino acid sequence of the coat surface proteins;
- (2) the presence of a start and a stop signal (indicating that the gene in its entirety has been obtained); and (3) the presence of either a C-terminal or an N-terminal amino acid.

5

Step 2. Fusing the candidate phage surface protein with the complement-antagonizing peptide of interest:

- a) Preparing the coat protein gene for fusion:

10

The gene for a surface protein is contained in its plasmid expression vector. The oligo-nucleotide for the complement-antagonizing peptide is spliced into this plasmid expression vector by in-frame fusion at the 3'-end of the coat surface protein.

15

- b) Incorporating the fusion gene into the phage of interest:

The fusion gene is incorporated into the phage by the in vivo generalized recombination system in the host bacteria for the phage.

20

- c) Demonstrating that the phage expresses the fusion protein:

The phage is incubated with a corresponding heavy metal-marked antibody that has been raised against the coat surface protein. The marker is detected on the phage by electronmicroscopy only if

25

the phage has expressed that fusion protein on its surface. [See e.g. K. Williams and M. Chase, Methods In Immunology and Immunochemistry, Vol.1, 1967, Academic Press.]

- 5 Example 5. Demonstration that the genetically engineered phage delay inactivation by the HDS, compared to wild-type phage:

Two groups of mice are injected with phage as specified below:

10 Group 1: The experimental group receives an IV injection consisting of  $1 \times 10^{12}$  of the genetically modified phage, suspended in 0.5cc of sterile normal saline.

15 Group 2: The control group receives an IV injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the genetically modified phage were derived, suspended in 5cc of sterile normal saline.

Both groups of mice are bled at regular intervals, and the blood samples assayed for phage content (by pfu assays) to determine the following:

- 20 1) Assays for half-lives of the two phages: For each group of mice, the point in time is noted at which there remains in circulation only half (i.e.,  $1 \times 10^6$ ) the amount of phage as administered at the outset. The point in time at which half of the genetically modified phage have been eliminated from the  
25 circulation is at least 15% longer than the corresponding point in time at which half of the wild-type phage have been eliminated from

the circulation.

2) Assays for absolute numbers: For each group of mice, a sample of blood is taken at precisely 1 hour after administration of the phage. The criterion used is that at 1 hour post-injection, pfu assays reveal that the numbers of genetically engineered phage still in circulation in the experimental animal are at least 10% higher than the numbers of wild-type phage still in circulation in the control animal.

Example 6. Determination that the genetically engineered phage has a greater capacity than wild type phage to prevent lethal infections in mice.

Part 1. Peritonitis Model:

An LD<sub>50</sub> dosage of E. coli is administered intraperitoneally (IP) to laboratory mice. The strain of E. coli used is one known to be lysed by the coliphage strain that has been genetically engineered. The treatment modality is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist of the following:

Group 1: The experimental group receives an IP injection consisting of  $1 \times 10^{12}$  of the genetically engineered lambda coliphage suspended in 2cc of sterile normal saline.

Group 2: A first control group receives an IP injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the genetically modified phage were developed, suspended in 2cc of

sterile normal saline.

Group 3: A second control group receives an IP injection of sterile normal saline.

Evidence that treatment with the genetically modified phage prevented the development of a lethal event in the peritonitis model is measured by using the following three criteria:

(1) Survival of the animal

(2) Bacterial counts: Samples of peritoneal fluid are withdrawn every 1/2 hour from the three groups of infected mice, and the rate of increase or decrease in E. coli colony counts in the three groups is noted

(3) Phage control: Using the samples of IP fluid withdrawn from the infected mice, the numbers of pfu of the genetically engineered phage versus the numbers of pfu of the wild-type phage are noted.

## Part 2. Bacteremia Model:

An LD<sub>50</sub> dosage of E. coli is administered intravenously (IV) to laboratory mice, where the strain of E. coli used is known to be lysed by the coliphage strain that was genetically engineered. The treatment modality (see below) is administered precisely 20 minutes after the bacteria are injected, but before the onset of symptoms. The treatment modalities consist of the following:

Group 1: The experimental group receives an IV injection consisting of  $1 \times 10^{12}$  of the genetically engineered lambda coliphage

suspended in 0.5cc of sterile normal saline.

Group 2: A first control group receives an IV injection consisting of  $1 \times 10^{12}$  of the wild-type phage from which the genetically engineered phage were developed, suspended in 0.5cc of sterile normal saline.

Group 3: A second control group receives an IV injection of 0.5cc of sterile normal saline.

Evidence that treatment with the genetically engineered phage prevented the development of a lethal event in the bacteremia model is measured using the following three criteria:

(1) Survival of the animal

(2) Bacterial counts: In the samples of blood that are withdrawn every 1/2 hour from the three groups of infected mice, the absolute numbers as well as the rate of increase or decrease in E. coli colony counts is noted, for each of those three groups.

(3) Phage counts: In the samples of blood withdrawn from the infected mice, the numbers of pfu of the genetically engineered phage and the numbers of pfu of the wild-type phage are noted.

We claim:

1. A method for treating an infectious disease caused by a bacteria, in an animal, comprising:

administering to an animal in need of such treatment, a lytic or non-lytic bacteriophage that is specific for said bacteria in a dosage effective to substantially eliminate the bacteria, wherein said bacteriophage has a genetically inheritable ability to delay inactivation by an animal's host defense system.

2. The method according to claim 1, wherein said bacteria is a drug resistant bacteria.

3. The method according to claim 1, wherein said animal is not a mammal.

4. The method according to claim 1, wherein said animal is a mammal.

5. The method according to claim 4, wherein said mammal is a human.

6. The method according to claim 1, wherein said bacteriophage has at least a 15% longer half-life than a corresponding wild-type phage.

7. The method according to claim 1, wherein the bacteriophage is obtained by anti-HDS selection (serial passage) of a mutagenized or non-mutagenized bacteriophage which is able to survive in an animal for a longer period than a corresponding wild-type bacteriophage.

8. The method according to claim 1, wherein the bacteria is selected from the group consisting of Mycobacteria, Staphylococci, Vibrio, Enterobacter, Enterococci, Escherichia, Haemophilus, Neisseria, Pseudomonas, Shigella, Serratia, Salmonella and Streptococci, and the bacteriophage can effectively lyse the bacteria.

9. The method according to claim 8, wherein the bacteria is selected from the group consisting of M. tuberculosis, M. avium-intracellulare and M. bovis.

10. The method according to claim 1, wherein the bacteriophage is administered by way of an aerosol to an animal's lungs.

11. The method according to claim 1, wherein the bacteriophage is administered at a dosage of about  $10^6$  to about  $10^{13}$  pfu/kg/day.

12. The method according to claim 11, wherein the bacteriophage is administered at a dosage of about  $10^{12}$  pfu/kg/day.

13. An isolated and purified bacteriophage that has a genetically inheritable ability to delay inactivation by an animal's host defense system.

14. The bacteriophage according to claim 13, wherein said bacteriophage has at least a 15% longer half-life than a corresponding wild-type phage.

15. The bacteriophage according to claim 13, wherein the bacteriophage is obtained by anti-HDS selection of a bacteriophage that is able to survive in an animal's body longer than the corresponding wild-type bacteriophage.

16. The bacteriophage according to claim 13, wherein the bacteriophage is obtained by genetic engineering of an anti-HDS bacteriophage that is able to survive in an animal's body longer than the corresponding wild-type bacteriophage.

17. The bacteriophage according to claim 13, wherein said phage is specific for bacterial families selected from the group consisting of Escherichia, Klebsiella, Shigella, Salmonella, Serratia, Yersinia, Enterobacter, Enterococci, Haemophilus, Mycobacteria, Neisseria, Pseudomonas, Staphylococci, Streptococci and Vibrio.

18. A method of obtaining a bacteriophage that is able to delay inactivation by an animal's host defense system against foreign bodies, comprising:

- (a) intravenously injecting a bacteriophage into an animal;



- (b) obtaining serial blood samples over time and measuring the bacteriophage present in each sample;
- (c) growing a portion of a sample obtained when about 0.1% to 1% of the bacteriophage remain in said animal, to high titer in a host bacteria; and
- (d) repeating steps (a), (b) and (c) at least once, to yield an "anti-HDS" bacteriophage that has delayed inactivation by an animal's host defense system.

19. The method according to claim 18, wherein step (d) is repeated until a bacteriophage is obtained which has at least a 15% longer half-life than a corresponding wild-type phage.

20. A method of producing a bacteriophage able to delay inactivation by an animal's host defense system, comprising genetically engineering a bacteriophage to express molecules on its surface coat that delay inactivation of the bacteriophage by an animal's host defense system.

21. The method according to claim 1, wherein the bacteriophage is obtained by genetic engineering.

22. The method according to claim 20, wherein the bacteria is selected from the group consisting of Mycobacteria, Staphylococci, Vibrio, Enterobacter, Enterococci, Escherichia, Haemophilus, Neisseria, Pseudomonas, Shigella, Serratia, Salmonella and Streptococci, and the bacteriophage can effectively lyse the bacteria.

23. The method according to claim 22, wherein the bacteria is selected from the group consisting of M. tuberculosis, M. avium-intracellulare and M. bovis.

24. The method according to claim 20, wherein the bacteriophage is administered by way of an aerosol to an

animal's lungs.

25. The method according to claim 20, wherein the bacteriophage is administered at a dosage of about  $10^6$  to about  $10^{13}$  pfu/kg/day.

26. The method according to claim 25, wherein the bacteriophage is administered at a dosage of about  $10^{12}$  pfu/kg/day.

27. A method for treating an infectious disease caused by a bacteria, comprising administering to an animal in need of such treatment an antibiotic and/or a chemotherapeutic agent in combination with a bacteriophage specific for said bacteria, in a dosage effective to substantially eliminate the bacteria, wherein said bacteriophage has a genetically inheritable ability to delay inactivation by the animal's host defense system.

28. A pharmaceutical composition comprising an isolated and purified bacteriophage which has a genetically inheritable ability to delay inactivation by an animal's host defense system, in combination with a pharmaceutically acceptable carrier.

29. The pharmaceutical composition according to claim 28, wherein said composition is an aerosol formulation for administration to an animal's lungs.

30. The pharmaceutical composition according to claim 28, wherein said bacteriophage is in lyophilized form.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/03930

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 7/00, 7/01, 15/01, 15/09

US CL : 435/172.3, 235.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 235.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, WORLD PATENT INDEX

search terms: phage, bacteriophage, serial passage, host defense

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Microbiology, Volume 128, issued 1982, H. Williams Smith et al, "Successful Treatment of Experimental <i>Escherichia coli</i> Infections in Mice Using Phage: its General Superiority over Antibiotics", pages 307-318, see entire article.	1-30
Y	Journal of General Microbiology, Volume 133, issued 1987, H. Williams Smith et al, "The Control of Experimental <i>Escherichia coli</i> Diarrhoea in Calves by Means of Bacteriophages", pages 1111-1126, see entire article.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A document defining the general state of the art which is not considered to be of particular relevance	* X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E earlier document published on or after the international filing date	* Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & document member of the same patent family
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* P document published prior to the international filing date but later than the priority date claimed	

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Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

NANCY T. VOGEL

Telephone No. (703) 308-0196

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